Genetically Modified Skin to Treat Disease: Potential and Limitations

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Molecular definition of disease at the level of the gene and advances in recombinant DNA technology suggest that many diseases are amenable to correction by genes not bearing the defective elements that result in disease. Many questions must be answered before this therapy can be used to correct chronic diseases. These questions fall into safety and efficacy categories. Experience with transplanting cellular elements of skin or skin substitutes (defined as skin that possess the cell types and a dermal structure to develop into a functioning skin) to athymic rodents is considerable and is seen as a system where these questions can be answered. This paper reviews these questions and presents our early analysis of genetically modified cells in skin substitutes *in vivo* and *in vitro*.

Experimental data demonstrate that both a matrix of woven nylon, housing a fibroblast generated collage, and dead dermis can be utilized to shuttle genetically modified human fibroblasts from the laboratory to an *in vivo* setting. Genetically modified fibroblasts do not migrate from the shuttle to the surrounding tissue. The survival of significant numbers, ~ 70%, of genetically modified fibroblasts for at least 6 weeks in these shuttles, supports this general approach as having clinical utility. It is also concluded that skin substitute systems can be used to generate a genetically modified skin *in vitro* that has the capacity to develop into functional skin *in vivo*. Further, as genetically modified keratinocytes differentiate there is increased production by the transgene, supporting the concept that keratinocytes have true potential as shuttles for therapeutic genes.

This work demonstrates that transplantation of systems containing genetically modified cells of the skin can be used to experimentally define many aspects of gene therapy using skin before this technology is taken to the clinic. Examples include determining the effect of gene transduction and expression on structure and function of the genetically modified skin as well as on distant skin and an assessment of the translational capacity of the transgene as function of time and cell number. *J Invest Dermatol 103:76S–84S, 1994*

Without resorting to hyperbole it can be stated that the status of health or disease inherent to an organ or organism at any given time is a result of the regulation of genes and their products. When this regulation is abnormal there are consequences that have patterns that physicians have learned to recognize as specific diseases. This is true whether the abnormality is via inheritance of an abnormal gene, infection with a microorganism, immune recognition of self, or mutational events that occur after birth.

Recent knowledge and methods from the field of recombinant DNA technology have made it increasingly possible to define health as well as disease at the level of the gene. This technology has generated molecular tools for mapping genes to specific locations on the chromosome, for nucleotide sequencing of normal and diseased genes, and for constructing plasmids that produce genes and their gene products *in vitro*. Over the past 10 years, application of this technology has also generated, at an ever increasing pace, specific genetic and molecular tests for disease, a molecular basis for physiology and pathology in health and disease as well as a rational basis for therapy. With these tools and the knowledge generated came the realization that corrected copies of defective or deleted genes might be placed in cells of patients to treat specific afflictions, i.e., gene therapy. This exciting possibility became reality in 1990 when the gene carrying adenosine deaminase was introduced into lymphocytes of patients suffering from severe combined immunodeficiency of childhood and the infusion of these genetically modified (GM) cells back into the patient [1].

This success has led to an explosion of gene therapy protocols for human subjects (43 approved by NIH and FDA approval boards by May of 1993) for a growing list of diseases. A partial list includes: placing the gene for CFTR (cystic fibrosis transmembrane conductance regulator) in the respiratory epithelium of patients afflicted

No reprints will be available.

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Abbreviations: β-chorionic gonadotrophin; β-hCG, human; CFTR, cystic fibrosis transmembrane conductance regulator; CMV, cytomegalovirus; DEDD, dead dermis; GM, genetically modified; GMF, genetically modified fibroblasts; GMK, genetically modified keratinocytes; HLDM, hypotonically lysed LDM; *LacZ*, β-galactosidase; LDM, living dermal model; MPS, mucopolysaccharide storage disorder.

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with cystic fibrosis; adding the gene for TNF-α to tumor infiltrating lymphocytes prior their expansion *in vitro* and injection back into cancer patients to augment tumor killing and placing genes in tumor cells to make them more immunogenic [2].

GENE THERAPY VIA SKIN/POTENTIAL

It has been obvious that skin should be an ideal organ for gene therapy, for some of the reasons listed below.

- Cells of the skin are readily obtained, cultured, cryopreserved, and passaged; making it possible to use autologous cells for genetic modification.
- A number of genes have been successfully transduced into cells of the skin; growth hormone [3], human β-chorionic gonadotropin [4], lacZ (see below) [5], factor VIII and IX [6,7,8], chloramphenicol acetyltransferase (CAT) [9], human insulin [10], human transferrin (unpublished observations Kaplan J, Morgan JR, University of Utah and Shriners Burn Institute, 1993), α 1-antitrypsin [11], human adenosine deaminase [12] and granulocyte–colony simulating factor [13].
- Systems for shuttling genetically modified (GM) fibroblasts and or keratinocytes from the laboratory to the patients are available and have undergone some clinical testing, see below.
- Symptoms of a mucopolysaccharide storage disorder in mice (MPS VII), analogous to human disease, have recently been corrected by transducing the human β-glucuronidase gene into MPS VII fibroblasts that were placed into a shuttle of collagen coated polytetra-fluoroethylene fibers (Teflon) and transplanted intraperitoneally [14].
- Procedures for the clinical transplantation of skin substitutes, (epidermis, split-thickness grafts, etc.) are routine.
- Skin is easy to examine for possible unwanted effects of gene therapy on the structure and function of skin
- If unwanted events occur in the GM skin, it can be removed.
- Blood supply to skin exceeds metabolic needs by ~10X. This feature should enhance the transport of therapeutic gene products from skin to the systemic circulation [15].

This paper focuses on the practical aspects of the potential, as well as the limitations, for correcting disease states using GM cells of the skin. Many important elements of gene therapy are not addressed, e.g., the disease or pathologic events of interest needs to be defined at the level of the gene; the gene of interest needs to be suited for replication in recombinant form *in vitro;* techniques are needed for placing the gene of interest into the cells that are to be transported to the patient; and methods are needed for promoting and controlling the gene transferred (transgene). The bibliography includes selected reviews of these important issues [1,16–21].

The major question is what types of disorders can be corrected by placing genes in cells of the skin? At least three general types of disorders appear amenable to gene therapy using cells of the skin.

The first is correcting systemic disorders with GM skin cells. Prototype diseases might be ones characterized by an abnormality in a plasma protein, such as hemophilia due to either factor VIII or IX deficiency [21] or α 1-antitrypsin deficiency associated with emphysema [11]. Other examples could be the impaired immune states inherent to diseases such as leprosy, tuberculosis, and AIDS. Here vectors that allow the selective over-production of a cytokine such as IFN-γ could be placed in cells of the skin to upregulate the more protective T_H1 response and to downregulate cytokines that drive T_H2 type responses, the latter being non-protective to the host in these diseases [22]. For these types of disorders the gene product would necessarily need to be transported from the site of generation in the skin to sites expressing disease. This aspect is covered in further detail in sections to follow.

The second group are skin disorders that are localized. At least two types of localized skin disease are potential targets; those caused by genetic defects and those that are autoimmune in nature. Candidate diseases in the former are: those with a defined gene defect, those where expression occurs later in life, those with localized areas of expression, and those where the heterozygous carriers are asymptomatic (i.e., one half or less of levels of a gene product will correct disease to the level of the asymptomatic carrier). In these diseases a corrected copy of the gene inserted into the element of skin (keratinocyte, fibroblast, melanocyte, etc.) most responsible for disease expression should result in a normalization of the disease. Because Darier's disease (keratosis follicularis) is inherited, presents late in childhood and frequently remains localized in expression, it is a candidate disease. Once the gene defect is delineated, placing a corrected copy of the gene into cells of the affected areas could do much to moderate disease. Other candidate skin diseases that have been or are in the process of being genetically defined, that are localized in expression, and that cause most of their morbidity later in life include several forms of epidermolysis bullosa, the basal cell nevus syndrome, and ultra-violet light induced squamous and basal cell carcinomas caused by mutations in p53 or other tumor genes [23,24]. Inherent to these considerations is whether such genetic diseases have to be corrected locally or can be corrected by genes at a remote site.

Correction of localized expression of autoimmune disease might be accomplished by several approaches. Examples include supplying a gene that alters trafficking of inflammatory cells, supplying a gene that upregulates suppression or downregulates antigen recognition moieties such as B7, or supplying a gene whose products (IL-4, IL-5, and IL-10 versus IL-2 and IFN-γ) respectively facilitate switching cells from T_H2 (non-opsonizing antibody phenotype production IgA, & IgE) to T_H1 (cell mediated and opsonizing Ig phenotype) phenotype [17,22]. Diseases approachable with these types of gene therapy include dermatitis herpetiformis, psoriasis, atopic dermatitis, localized scleroderma, and discoid lupus erythematosus.

The third group represent a broader perspective, correcting generalized skin disorders, either auto immune (e.g., generalized scleroderma) or genetic (e.g., epidermolytic hyperkeratosis, lamellar ichthyosis, or mucopolysaccharide storage disorders) with gene therapy using cells of the skin. The principles are the same, however the candidate gene or its product needs to be delivered from localized sites to the entire skin and to other organs if more than the skin is affected. These localized sites could carry and express the normal gene or house genes that can regulate expression of genes that give rise to disease, e.g., autoimmune processes.

GENE THERAPY VIA SKIN/LIMITATIONS

For each of the forgoing examples using genetically altered skin to correct disease, there are a series of questions that serve to define the limitations of this promising form of therapy.

Which Cell(s) Should Be Used to Shuttle the Therapeutic Gene to the Skin? If the defect is localized, and selectively expressed by elements in the skin, e.g., epidermis, it is intuitive that the gene be placed in keratinocytes. Because the epidermis does not have an active blood supply, expression of the transgene in the dermis seems most appropriate where transport to systemic circulation is required. However, this may be only theoretical. Recently Fenjves *et al,* in association with our group, have shown that human apolipoprotein-E made by keratinocytes is present in the sera of nude rats bearing human skin grafts on the rat sandwich flap system [25]. Higher levels were noted in blood draining the flap bearing the grafts than in the systemic circulation, (13.8 ± 0.2 ng/ml versus 8.8 ± 0.3 ng/ml, respectively). Thus, the lack of an active blood supply is less of a concern. In actuality no cell has a direct blood supply, all having at least a cell membrane and/or other matrix elements between the site of production and blood.

Are There Limitations in the Number of Cells That can be Made to Express the Transgene in a Given Tissue? It is plausible that if every cell carries one or more copies of the transgene an alteration in structure and function of skin could develop. Studies of over-production of factors normally made by cells of the skin have been and are being conducted in transgenic mice. Some have caused substantive changes, (e.g., over-expression of TGF-β_1 causes a taut skin with an increased stratum corneum and diminished number of hair follicles in affected pups) [26]. The number of genetically al-

tered cells needed to produce this effect is unknown. For every cell to carry the transgene, cells have to be cloned after transduction or transduction has to be at 100% efficiency.

Will the Product of the Transgene be Immunogenic or Induce an Immune Response to the GM Cells? It is presumed that a gene product that is endogenous to the healthy counterpart will not be immunogenic when expressed in patients. However, there is evidence to indicate that human factor IX made by GM murine fibroblasts can be immunogenic when transplanted to syngeneic mice [5].

What are the Limits of Production by the Transgene in GM Skin, i.e., Is There a Dose Response and Does the Gene Product Have a Shortened Half-Life? A recent report notes that human factor VIII has a much abbreviated half-life in mice bearing GM murine fibroblasts [6].

Is the Transgene Stable, Does it Become Inactivated as a Function of Time, Does the Cell Bearing the Transgene Become Senescent, Is the Cell Bearing the Transgene Rejected [5,12,16,18]?

Will the Genetic Alteration Activate Proto-Oncogenes and Cause Tumors to Form, Will the Transgene Alter Homeostasis Sufficient to Cause Abnormal Structure, and/or Function in Skin Carrying the Genetic Modification? There are no known reports of this occurring.

Is the Transgene Product Intended to Have Autocrine, Paracrine, or Endocrine Effects, i.e, Will the Gene Product Leave the Site of Origin? The answer will govern the cell type used.

What are the Most Clinically Relevant Methods for Generating GM Skin? It is appreciated that need directs choice, the broad choices are 1) a matrix carrying GM fibroblasts buried subcutaneously to deliver gene products to the systemic circulation or placed intradermally for delivery to the surrounding dermis or overlying epidermis; 2) a skin substitute, defined as a matrix carrying normal or GM fibroblasts with the potential for developing an epidermis from normal or GM keratinocytes seeded on the surface; 3) GM keratinocytes to replace an epidermis that has been removed. The concerns are how to remove the existing epidermis, when and how to replace it with the GM elements, and whether follicular keratinocytes of the host will gradually replace the GM element.
There are a number of dermal matrices that are readily impregnated with fibroblasts that can also be seeded with keratinocytes: 1) a nylon matrix dermal model (see below) implanted with fibroblasts that generate collagen that fills the matrix and surrounds the nylon fibers, (Advanced Tissue Sciences, La Jolla, CA) [27–29]; 2) a dead dermis (DeDD, see below) generated from human skin [30,31]; 3) a collagen gel to which fibroblasts are added de novo or in combination with a support structure such as polytetra-fluoroethylene fibers (Teflon) [14,28,32–34); 4) a mesh generated from polyglycolic acid (Vicryl) impregnated with fibroblasts (Living Dermagraft, Advanced Tissue Sciences, La Jolla, CA); and 5) a collagen-glycosaminoglycan dermal substrate with or without a laminated sheet of collagen on one surface for attachment of keratinocytes [35–37].
The first two do not undergo biodegradation in the short term (unpublished observations of transplants to nude mice for more than two months); the other three have potential to biodegrade. There are no studies that compare biodegradability of a shuttle with its efficacy to move GM cells to an *in vivo* setting.

Are Stem Cells Needed? Short term (weeks to months) survival of primary GM fibroblasts has been demonstrated in several *in vivo* settings [5,12–14,16]. There are no studies that show that the GM cells persist for the life of the host. It is accepted that primary cultures of keratinocytes and fibroblasts undergo senescence *in vitro*. It is presumed that this also occurs *in vivo*. Thus, the time that a cell carrying a transgene in its genomic DNA would be therapeutic is proportional to the number of progeny and the life span of each. In the absence of active shut-down by host tissue, the length of time that a transgene or its message is operational after cell division ceases is unknown. Because skin survives despite age-associated senescence *in vivo*, it is accepted that stem cells (cells possessing unlimited renewal capacity) exist in skin. Placing the transgene in the genomic DNA of a stem cell would hypothetically circumvent this potential cause of loss of therapeutic effect. However, to date, the source and nature of stem cells in skin have not been identified for human skin. It should be noted that predicted shut-down could be used in selected settings, e.g., situations requiring short-term gene therapy, such as treatment of an acute illness.

Proliferative events will predominate over differentiative events for some time after implanting GM tissue. With differentiation the typical cell shifts to the manufacture of products that lead to its function. Thus, unless regulatory elements in the vector dictate otherwise, it is predicted that GM cells will produce more gene product as proliferation returns to baseline and differentiation becomes dominant. This hypothesis is supported by the observations that keratinocytes express more of the transgene (CAT) with differentiation [9]. Whether these biologic observations and conjectures are uniformly operative and in harmony with the promotors used with the transgenes and with the concept of random insertion of the transgene into host genomic DNA is to be determined.

What is the Best Method to Deliver the Selected Gene? Several methods have been used to introduce genes into cells and or hosts [16,18,20,38–40]. 1) One method is physico-chemical, e.g., $CaPO_4$, DEAE-dextran, liposomes, etc. 2) Another is micro injection into cells or tissue, e.g., muscle. 3) There is electroporation or Gene Gun (DNA-coated gold beads). 4) In ligand directed delivery, DNA (transgene) is attached to a ligand and delivered to cells via the receptor for the ligand. This model suggests it is possible to transport a transgene attached to a ligand via the bloodstream to a specific tissue with tissue specific receptors or modified to express binding sites for the ligand. Work by Wu and Wu [41], using a binding site for asialoglycoprotein, demonstrates feasibility for using this technique to deliver transgenes to the liver. Another example might be the induction of over-expression of class II MHC antigen in the epidermis with INF-γ, conjugating the transgene to an antibody for the specific MHC antigen and injecting it intravenously. 5) In viral directed delivery, viruses are used because of their selective ability to enter cells. Types of viruses under consideration include the retrovirus, adenovirus, adeno-associated virus, herpes virus, HIV, and papovavirus. For reasons listed in the following paragraph the majority of experience in gene transfer has been with retroviruses [16,18,20].

The first three transduction methods on this list are generally quite inefficient, $<1\%$ of cells being successfully transduced. This contrasts with transduction with retrovirus technology where transduction efficiencies $>20\%$ are common. Additional advantages of retroviruses for gene transduction include 1) they have amphotropic host range, i.e., will infect a variety of cells, including human; 2) stocks of recombinant retroviruses that are free of wild-type replication-competent virus can be produced from specialized packaging cell lines; 3) their integration into host genomic DNA is efficient and provides a stable provirus with predictable structure.

Transduction with adenovirus is also very efficient as large numbers of cells can be made to carry multiple copies of the transgene [18]. However, the transgene is episomal (i.e., it does not incorporate into host genomic DNA) and hence will have a limited life span.

Can GM Cells of the Skin Be Used to Correct a Relevant Disorder in a Rodent Model? There is at least one rodent model of disease where gene therapy has been used. In the mouse model of an inherited mucopolysaccharide storage disorder (MPS

Table I. Predicted Production of Gene Product by Cells of Skin

Type of GM Tissue	Tissue Volume $\mu m^3 \times 10^{10}$	Number of Cells $\times 10^7$	Production in ng/h
Skin equivalent with GM keratinocytes & GM fibroblasts			
Graft on rat flap (2 × 3 cm × 150 μm)	9	9	450–1800
Graft on mouse (2 × 2 cm × 150 μm)	4.7	4.7	235–940
Skin with GM epidermis			
On rat flap (2 × 3 cm × 150 μm)	3	3	3150–600
On mouse (2 × 2 cm × 150 μm)	1.6	1.6	164–256
Skin with GM dermis			
Implant (2 × 2 cm × 500 μm)	20	20	1000–4000
Implant (2 × 2 cm × 1000 μm)	160	160	8000–32000

VII) many symptoms were corrected by transplanting fibroblasts bearing the human β-glucuronidase gene to affected mice [14].

PREDICTED PRODUCTION BY GM SKIN

Factors affecting the potential for correcting genetic or acquired disorders with GM skin are 1) the number of GM cells, 2) the inherent production rate by the transgene, 3) degradation and/or elimination of gene product(s), 4) presence of receptors for the gene product, 5) solubility of the gene product in the tissue within which it is generated in, 6) blood flow to this tissue, and 7) solubility in blood. We have calculated the number of GM keratinocytes (GMK) or GM fibroblasts (GMK) that can be carried in the various types of grafts that can be transplanted to athymic rodents. These are the number of GMD contained in a GM epidermis that would replace the existing epidermis of an orthotopically engrafted human split-thickness skin graft; the number of GMF carried in an artificial dermal matrix that would be placed in the subcutaneous tissue; and the number of GMK in the epidermis and the GMF in the dermis of a skin substitute. From this we have calculated the potential rate of production by these GM tissues using currently available promoters (see **Table I**). These calculations support the contention that some, perhaps many, heritable disorders can be corrected with GM skin. These calculations assume the following: 1) Kc are approximately 1000 μm^3 in volume, 10 μm on a side [15] and give rise to the epidermis which is ~50 μm thick; 2) The number of fibroblasts in a dermal matrix, ~300 μm thick, is equivalent to the number of keratinocytes in a like area of the overlying epidermis; 3) In a human skin graft on the rat human skin sandwich flap, the epidermis can easily measure 2 cm × 3 cm × 150 μm in thickness, a volume of 9×10^{10} μm^3. An orthotopic human skin graft on a mouse typically can measure 2 cm × 2 cm in diameter × 150 μm in thickness, 4.7×10^{10} μm^3 [42]; 4) In orthotopically grafted skin that is re-epithelialized with GMK, the productive epidermal volume is 3×10^{10} μm^3 and 1.57×10^{10} μm^3 for the rat flap and the mouse graft, respectively; 5) a dermal matrix 1 mm in thickness and 4 × 4 cm in size has a volume of 1.6×10^{12} μm^3; 6) the number of GM cells in each of the combinations equals volume of tissue/volume of cells (all cells are assumed to be 1,000 μm^3, an overestimate of the size of fibroblasts and extracellular space is ignored; 7) expansion of tissue or death of cells during and after engraftment likely will occur, this has not been included in these estimates; 8) with current retroviral vectors the rate of production by the transgene of GM cells is ~5–20 ng/10^6 cells/h, this presumes all cells carry the gene (Jeff Morgan, personal communication, 1993); 9) production equals number of cells × the rate × time; 10) blood levels can be predicted with the formula $P = \frac{\ln 2}{t_{\frac{1}{2}}} \times (X)$, where P, the production rate in ng/h; X, the level of protein in the blood, in ng/ml, × the total plasma volume; average 70 kg man, 2700 ml; ln2, the natural log of 2; $t_{\frac{1}{2}}$, the half life of the protein in hours.

This analysis suggests that the range for GM skin to correct disease is considerable. These estimates and the progress noted in the forgoing sections make it probable that a new field of pharmacology will emerge, the pharmacology of genes and gene products, it will encompass parameters that define production, (e.g., promotors, number of gene copies per cell, etc); parameters that define transport in tissue, size, charge, solubility, etc; solubility in tissue and blood; pharmacokinetics of gene products *in situ*, $t_{\frac{1}{2}}$ in tissue versus blood; and pharmacodynamics, dose/response.

PROGRESS TOWARD USE OF GENETICALLY MODIFIED (GM) CELLS OF THE SKIN TO CORRECT DISEASE

Recently we initiated work that is directed at determining the feasibility of using GM cells of human skin to correct disease by analyzing the GM elements *in vitro* and *in vivo* after transplantation to athymic rodents. There are three long term objectives. First, determine the long-term functionality of skin containing GM components in an *in vivo* setting, (specifically, the stability of the transgene and production of its product, the aspects that define a dose response and the effect of the transgene on structure and function inherent to skin). Second, develop the necessary technology for transplanting GM skin to humans using transplantation of GM skin to athymic rodents as a developmental intermediary, (specifically, an approach to replace the existing epidermis with an epidermis generated from GM keratinocytes and generating a GM skin-equivalent that carries normal or GM fibroblasts or keratinocytes that can be transplanted onto athymic rodents). Third, determine whether disease can be corrected with keratinocytes and or fibroblasts transduced with the appropriate gene. The remainder of this manuscript reports relevant aspects of progress on the first objective.

MATERIALS AND METHODS

The athymic (nude) mice supplied by Charles River, Wilmington, MA., used in these experiments were BALB/c, male, and ~3 months of age at the time of grafting.

Two dermal substrates to shuttle GM cells to nude mice have been used in these studies. The first is the living dermal model (LDM) supplied by Advanced Tissue Sciences, La Jolla, CA. This substrate is a nylon mesh that is treated to promote fibroblast adhesion, seeded with foreskin fibroblasts at Advanced Tissue Sciences, held under tissue culture conditions for three weeks and incubated with media selective for fibroblasts supplemented with ascorbic acid. During this time the interstices of the mesh fill with fibroblasts and collagen. Thereafter the LDM is cut into 1.3 cm × 1.3 cm squares, subjected to hypotonic lysis (HLDM) to kill the fibroblasts and shipped to our laboratory where it is stored at −70°C in DMEM with 10% fetal bovine serum (FBS).

The second dermal substrate is dead dermis (DeDD) that is generated from human skin using a procedure modified from Langdon and McGuire [30]. Within 4 h of surgery remnant skin from elective abdominoplasties is delivered to our lab in containers filled with saline supplemented with antibiotics and a fungicide. It is keratomed to 500 μm thickness with a Padgett Electro Dermatome, Kansas City, MO., placed in a 56 to 60°C water bath for 30 seconds and the epidermis peeled away with forceps. The DeDD is subjected to 3 freeze (−70°C) thaw (24°C) cycles at 24 h intervals, irradiated with a cesium source (5000 cGy), cut into 1.5 cm × 1.5 cm pieces and stored at −70°C.

Histologic analyses of the HLDM and the DeDD demonstrate nuclear debris without evidence of viable cells. This is supported by finding no cells on tissue culture plates housing the HLDM or the DeDD when held under tissue culture conditions (DMEM with 10% FBS and 10% CO_2 at 37°C) for prolonged periods, more than 30 d.

Cell types used in these experiments include a NIH 3T3 cell line, three different human fibroblast sources, neonatal (two), generated from fore-

skins, abbreviated FsF and adult (one), generated from abdominoplasty skin, abbreviated AdF. To establish these fibroblast cultures the skin was dissociated with trypsin, fibroblasts collected and seeded at relatively high density, grown to confluence, harvested, expanded, and passed × 3, frozen, and stored in liquid nitrogen. After expansion the 3T3 line was stored in liquid nitrogen.

In skin substitute experiments the dermal substrates were seeded with neonatal fibroblasts (1×10^6) at day 0 and held in culture for 14 d; on day 15 neonatal keratinocytes [$1 \times 10^6/50\ \mu l$ of KGM (keratinocyte growth media), Clonetics, San Diego, CA] were seeded on the surface. On day 16 these skin substitutes were transplanted to the subcutaneous space of the lateral thoracic cage of nude mice. Wounds were closed with wound clips, which were removed 10–14 d later. Grafts were excised at periodic intervals for histologic assessment.

Two retroviral vectors encoding the gene for lacZ from *Escherichi coli* and the gene for human β-chorionic gonadotropin (β-hCG) have been used. The former is termed α-SCG-lacZ and houses the promoter from CMV for α-globin. It is used with the permission of Somatix Therapy Corporation, Alameda, CA [43]. The latter is a retrovirus encoding the β-subunit of human chorionic gonadotropin. This vector, termed KSG β-hCG, has a promoter fragment and upstream sequences from a pseudogene of K14, a 50-KDa keratin protein expressed at high levels in keratinocytes and an efficient packaging sequence that increases the frequency of transduction of keratinocytes [4]. These vectors have been transfected into the 3T3 packaging cell line ψ-CRIP [44]. The 3T3 transfected lacZ cell line (3T3.T.lacZ producer) was expanded, tested for viral titer, frozen, and stored in liquid nitrogen. The 3T3 β-hCG producer was used to transduce foreskin keratinocytes shipped to Utah, expanded, and seeded (at $8 \times 10^5/cm^2$) on a fibroblast impregnated collagen-glycosaminoglycan dermal substrate with a laminated sheet of collagen on one surface for attachment of keratinocytes [36]. Culture conditions, a 60:40 mix of KGM and DMEM with 10% FBS [45], supported fibroblast growth as well as keratinocyte proliferation and differentiation. Every 72 h spent media was collected, β-hCG measured by ELISA, and new media added.

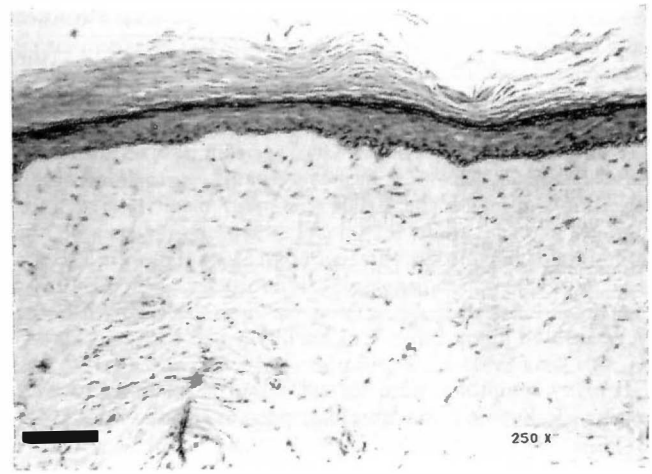
The supernatent from the 3T3.T.lacZ producer line was used to transduce 3T3 cells and fibroblasts from human sources. Transduction with virus carrying lacZ followed the format of splitting the cells to be infected on day 0, passing them at passage density, at day 1 adding undiluted fresh or thawed lacZ virus from the producer line with polybrene (4 μg/ml) and returning cells to the incubator with gentle shaking every 30 min. After 2 h media was withdrawn, new media added, and cells returned to the incubator at 37°C and 10% CO_2. When cells reached confluence, 3–5 d later, media was removed, plates fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature, and stained for lacZ with X-gal. LacZ remains in the cytoplasm; cells expressing lacZ stain blue after incubation with the substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) [46]. The efficiency of transduction is measured by staining for lacZ using X-gal and estimating the fraction of positive cells microscopically. For the 3T3 cells this ranged from 50–60%, for adult fibroblasts from 1–25% and for neonatal fibroblasts from 10–40%.

The lacZ transduced cells (3T3, adult fibroblasts, neonatal fibroblasts) were expanded by growing to confluence, harvesting, splitting, and passage × 3. One million of the lacZ transduced cells were added to the upper surface of freshly thawed squares of HLDM or DeDD in a volume of media that did not let fluid spill over the edge of the HLDM (~ 50 μl). These combinations were held *in vitro* for up to 28 d or were transplanted to the subcutaneous space of the lateral thoracic cage of nude mice at day 8. Wounds were closed with wound clips. Transplants were removed at 2 week intervals for 6 weeks and analyzed for lacZ expression using the X-gal staining procedure [46]. The HLDM requires plastic embedding and 5 μm sections for optimal histologic assessment. Staining whole tissue with X-gal results in surface staining only, thus tissue needs to be trimmed to a 1 to 2 mm thickness before staining for lacZ. The X-gal stain survives the tissue sectioning process. Sections were counter-stained with hematoxylin and the number of positive cells estimated on a 0 to 4 scale where 0 = none and 4 = all cells as positive for LacZ. The procedure for the DeDD was the same only it was submitted for routine tissue sectioning.

The need to know the fate and effect of GM cells in or on these substrates when all cells carry the transgene caused us to clone GMF, using the limiting dilution technique. This cloning utilized the neonatal foreskin fibroblast source that was most efficiently transduced with the viral supernatent carrying lacZ. Two clones emerged, one did not passage and one did. It was placed on the HLDM as noted above and handled in a manner analogous to the non-cloned component.

RESULTS

Transplant of Dermal Substrates Seeded with Keratinocytes and Fibroblasts to Nude Mice It was conjectured that a shuttle system to carry GM cells to the patient needs to have a demonstrated capacity to house the basic elements for a skin substitute (epidermis and dermis) *in vitro* and the potential to evolve into a functional epidermis and dermis *in vivo*. Thus an initial objective was to demonstrate that the dermal substrates, the dead dermis (DeDD), and the hypotonically-lysed dermal model (HLDM), could be seeded with keratinocytes and fibroblasts and transplanted to nude mice to evolve into a skin substitute (defined as skin containing a defined source of keratinocytes, fibroblasts, and dermal matrix that has the potential to function as normal skin).



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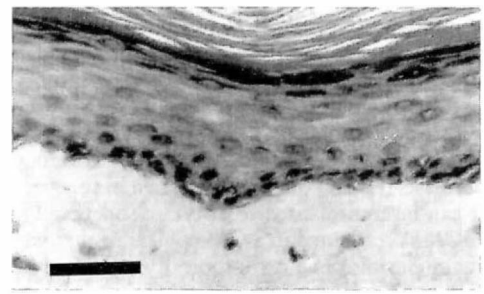

B

Figure 1. Representative photomicrographs (*A, bar* = 100 μm) and (*B, bar* = 50 μm) of a dead dermis (DeDD) 6 weeks after transplantation to the subcutaneous space of athymic (nude) mice. Prior to transplantation the DeDD was seeded *in vitro* with human fibroblasts at day 0. At day 15 human keratinocytes were added to the surface and the resultant skin substitute transplanted on day 16. Note the density of fibroblasts and the normal appearance of the epidermis.

Histologic analysis of DeDD grafts, seeded with keratinocytes and fibroblasts, at days 14, 26, and 35 demonstrated five of eight grafts with an intact epidermis and fibroblasts throughout the DeDD, **(Fig 1)**. Analysis of the HLDM revealed an immature epidermis overlying a fibroblast impregnated matrix **(Fig 2)**. When left air exposed the HLDM skin quickly desiccates and becomes nonviable. No inflammatory nor foreign body response was noted in tissue surrounding either skin substitute. Transplanting a fibroblast free HLDM demonstrated the density of mouse fibroblasts that move into this dermal substrate equals that of a LDM that was not lysed hypotonically and achieves the fibroblast density noted in the HLDM seeded with fibroblasts, noted in **Fig 2**. These features show that these systems have the potential to answer many of the questions associated with the technology of correcting diseases using GM keratinocytes and or fibroblasts.

Genetically Modified 3T3 Cells Seeded Unto Dermal Matrices 3T3.T.lacZ seeded onto a HLDM demonstrated that there was sufficient migration into the collagen by day 8 to permit subcu-

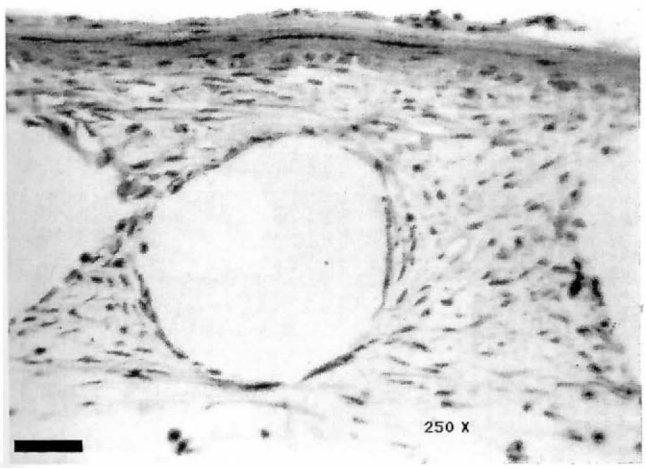

Figure 2. A representative photomicrograph (bar, 100 μm) of a hypotonically-lysed living dermal equivalent (HLDM) 6 weeks after transplantation to the subcutaneous space of athymic (nude) mice. Prior to transplantation the HLDM was seeded *in vitro* with human fibroblasts at day 0. At day 15 human keratinocytes were added to the surface and the resultant skin substitute transplanted on day 16. Note the density of fibroblasts and the immature appearing epidermis.

taneous implantation. Holding them *in vitro* for additional time revealed increasing numbers of cells through day 20, with a decline thereafter.

When transplanted to athymic mice, the 3T3.T.lacZ in HLDM developed into tumors. These were readily apparent by week 3, and by week 8 were sufficiently large to cause us to sacrifice the animals. Grossly, these tumors stained positive for lacZ using the X-gal staining technique. A similar analysis was accomplished with 3T3.T.lacZ seeded on DeDD. Migration of 3T3 cells into the DeDD did not occur. However, the cells did grow on the surface. Microscopic analysis of these tumors at 2, 6, and 8 weeks demonstrated many cells positive for the lacZ gene **(Fig 3)**. However, the percent evidencing this gene product were fewer *in vivo* than in the early days *in vitro*. Reasons for this decrease include: the GM cells may be at some selective growth disadvantage relative to the non-GM cells, there is mutational inactivation of the LacZ gene, or gene inactivation via methylation. Cloning by limiting dilution of 3T3.T.lacZ reveals clones where all cells positive for lacZ before the cells reach confluence. At confluence it is common to find areas where lacZ is no longer expressed. This suggests that lacZ is somehow inactivated at high density (confluence) *in vitro*. The *in vivo* corollary experiments have not been performed.

The fact that 3T3.T.lacZ gradually form tumors in and around these dermal substrates on nude mice is likely to be a useful tool for assessing gene product levels in blood vs. number of cells carrying the transgene as a function of time, i.e. time–dose responses.

Genetically Modified AdF and FsF to Dermal Matrices The analysis of lacZ survival in AdF transduced with lacZ and FsF transduced with lacZ after seeding them onto HLDM or DeDD, holding them *in vitro* for 8 d and then transplanting them subcutaneously to athymic mice demonstrate 1) GM fibroblasts readily move into the HLDM; 2) GM fibroblasts surface seed onto DeDD and increase through day 16, but do not move into the dermis; 3) *in vivo,* at days 14, 18, and 42, all three cell lines had gross and microscopic evidence of lacZ in both the HLDM and DeDD. However, by day 42 this was much more in evidence with the HLDM than with the DeDD; 4) the percent of cells demonstrating the lacZ gene is less *in vivo* than in the early stages *in vitro;* and 5) no inflammatory infiltrate is seen around either the HLDM or the DeDD implants bearing GM fibroblasts.

Cloned Genetically Modified FsF to Dermal Matrices The apparent depletion of GM cells as a function of time caused us to

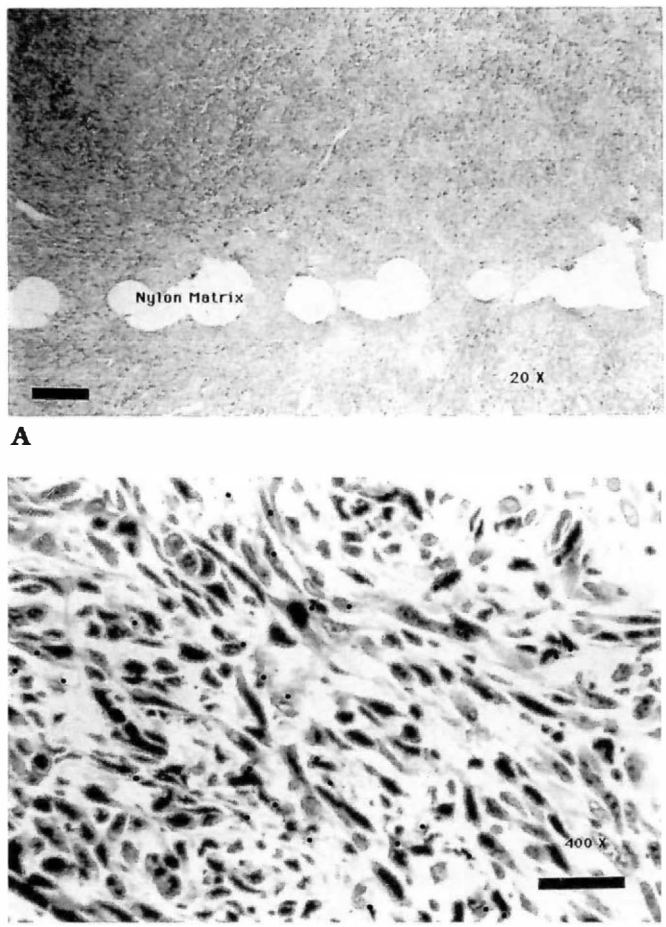

Figure 3. A low (*bar,* 300 μm) and high-power (*bar,* 50 μm) photomicrograph of a HLDM seeded with 3T3.T.lacZ, held *in vitro* for 8 d, grafted to athymic (nude) mice, and removed after various times of engraftment. These photomicrographs were taken of a tumor (1.5 cm × 1,5 cm × 0.8 cm) of a 3T3.T.lacZ HLDM graft after 6 weeks of engraftment. Only part of the tumor arising from the nylon matrix can be appreciated at low power. The number of cells that are lacZ positive are marked with a *(bullet)* in the high-power photograph (blue staining lacZ positive cells cannot be distinguished in a black and white photo from background hematoxylin staining).

clone, via limiting dilution, a normal human neonatal fibroblast source transduced with lacZ. HLDM and DeDD were seeded as above with this clone and analyzed *in vivo* and *in vitro*. These experiments demonstrate the following:

1. It is possible to clone human fibroblasts that have been transduced with a gene in a retroviral vector, expand same *in vitro,* and carry it to an *in vivo* setting with a dermal substrate.
2. Cloned fibroblasts move into the internal aspects of the HLDM within 4 d, where they increase through day 8 **(Fig 4)**; this contrasts with the surface where they continue to increase through day 21 before stabilizing.
3. The density of GM fibroblasts within the collagen matrix of HLDM was that seen in non-hypotonically lysed HLDM *in vitro* and in HLDM placed *in vivo* without fibroblasts (see above).
4. All cells in the HLDM *in vitro* were positive for lacZ.
5. Removal at days 14, 28, and 42 and staining with X-gal reveals most cells in the HLDM bear lacZ and that the DeDD also has many cells bearing lacZ **(Fig 5)**.
6. By day 42, versus day 14, there is an ~30% reduction in the number of fibroblasts bearing the lacZ gene in the HLDM. It

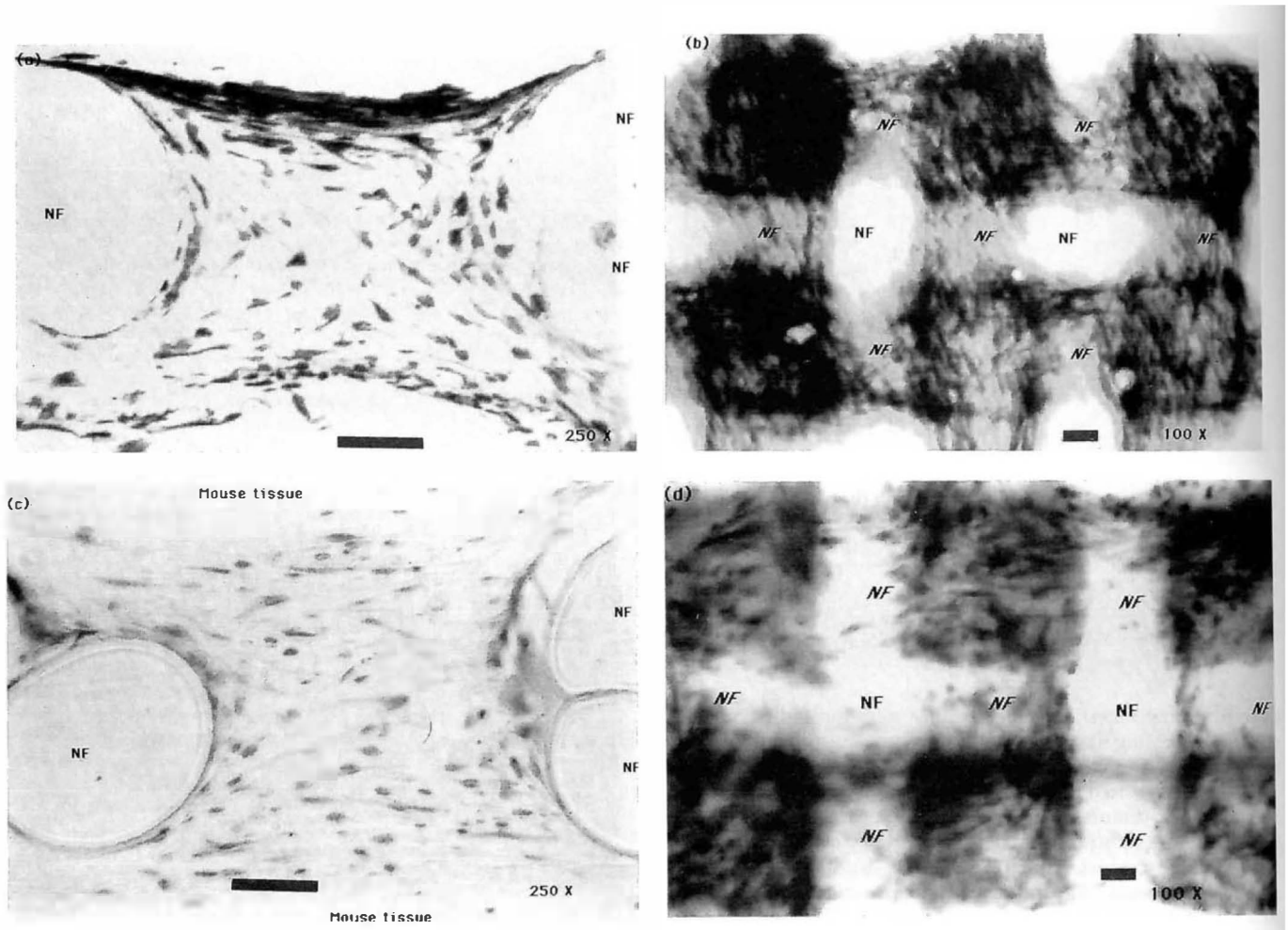

Figure 4. Photomicrographs of HLDM seeded with an expanded clone of lacZ-positive neonatal foreskin fibroblasts after 8 d *in vitro (a, b)* and after 42 d *in vivo (c, d)*. Cross sectional views *(a, c)* and *en face* views *(b, d)* are presented. NF and *NF* represent the crossing of the nylon fibers of the HLDM, (NF labels intersection in fibers, double fibers, *NF* labels the non-intersecting, single fiber, and no label is where the collagen has been laid down by human fibroblast between the nylon fibers before the LacZ fibroblasts were added. 1) All of the dark staining cells are those expressing lacZ, 2) the interstices of the matrix are filled to capacity, 3) the lacZ cells are confined to the HDLM, 4) cells in mouse tissue *(d)* do not stain for lacZ and thus are not visible, 5) the absence of an inflammatory infiltrate, or foreign body reaction. Size representation for **Fig 4** *(a)* through *(d)*; *bar,* 100 μm.

appears that normal mouse fibroblasts move into the HLDM and replace or displace the cloned GM fibroblasts as a function of time **(Fig 4)**. Whether this is because of senescence of the cloned GM fibroblasts or because the host fibroblasts are more aggressive is unknown.

7. In contrast with the experiment using 3T3.T.lacZ cells, there is no expansion of fibroblasts into the surrounding subcutaneous tissue of the mouse with either shuttle system.
8. No foreign body reaction is noted with either shuttle seeded with the cloned cells.

These experiments demonstrate that both the DeDD and the HLDM can be utilized to shuttle GM fibroblasts from the laboratory to an *in vivo* setting and that the HLDM appears to be more efficient. The observation that the GM fibroblasts did not leave the constraints of the shuttle suggests that the GM cells (normal fibroblasts) stay where they are placed. The survival of significant numbers, ~70%, of GM fibroblasts for at least 6 weeks, demonstrates a clinical utility for transplanting a dermal matrix containing GM cells. Histologic analysis of a DeDD after it has been transplanted to the nude mouse for more than six weeks shows that it has a greater capacity for GM fibroblasts than the HLDM. The number of fibroblasts that can take up occupancy in the DeDD *in vivo* is approximately three times that of the HDLM. For this reason experimental approaches are being designed to enhance the number of GM cells that move into the DeDD before being transplanted.

Production of β-hCG by Neonatal Keratinocytes *In Vitro*

The ease of obtaining, culturing, and replacing keratinocytes from and on patients makes them attractive as a shuttle for therapeutic genes. A preliminary assessment of the potential for production of a therapeutic gene by GM keratinocytes has been made using β-hCG transduced keratinocytes grown over a fibroblast impregnated dermal matrix, (see *materials and methods*). Levels of β-hCG increased throughout the length of the 26 d experiment, **(Fig 6)**. Media was changed about every 3 d and β-hCG measured by ELISA. By definition, after each media change there is essentially no β-hCG in the media. Nevertheless, with each media change, there was more product than at the previous media change, peak levels of 255 milinternational units (miu)/ml being achieved by the last day (day 26) of the experiment. At day 26, the skin-equivalent was harvested and subjected to histologic assessment. This revealed that the epidermis was composed almost totally of enucleated keratinocytes, i.e., a thin stratum corneum. This supports the notion that with increased differentiation there is increased production of transduced gene product and supports the observation of increased expression of CAT in keratinocytes as a function of differentiation [9]. Production by differentiated cells is important to the feasibility of using GM keratinocytes to treat disease as all keratinocytes have limited life spans. To avoid having to replace the GM cells at short intervals GM cells need to produce gene product in a differentiated state as long as possible. This potential problem could be overcome, in part, by transducing the gene into stem cells.

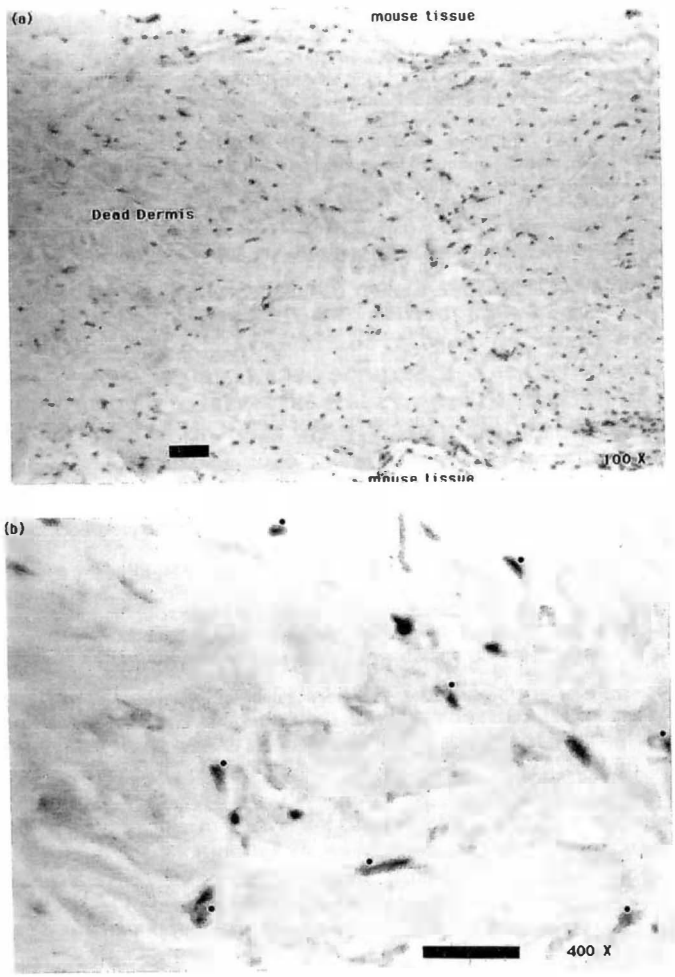

Figure 5. Low (*a*, *bar* = 100 μm) and high-power (*b*, *bar* = 50 μm) photomicrographs of DeDD seeded with a clone of lacZ-positive neonatal foreskin fibroblasts 42 d after subcutaneous engraftment to athymic mice. 1) LacZ cells, identified (by *bullets*) in *b*, are not uniformly present in this substrate that has been counterstained with hematoxylin, 2) note the absence of an inflammatory infiltrate or foreign body reaction, 3) there is no tissue necrosis, 4) the density of fibroblasts is equivalent to that of a normal human dermis, 5) the space in this dermal substrate to hold GM fibroblasts is greater in the HLDM (see **Fig 4**).

CONCLUSIONS

These experiments demonstrate that both the HLDM and the DeDD can be utilized to shuttle GM fibroblasts from the laboratory to an *in vivo* setting. Importantly GM fibroblasts do not migrate from the structure to the surrounding tissue. The survival of significant numbers of GM fibroblasts for at least 6 weeks in these shuttles, supports this general approach as having clinical utility.

The increased production by the transgene with increased differentiation and the observation that human apolipoprotein-E, made by keratinocytes, appears in the systemic circulation of animals bearing human skin grafts adds grist to the concept that keratinocytes have true potential as shuttles for therapeutic genes. The limitations await *in vivo* assessment.

It is also concluded that skin substitute systems will permit the generation of a GM skin *in vitro* and that they will go on to develop as functional skin *in vivo* after transplantation to the athymic rodent. With this we hope to gain answers to many of the questions that are central to understanding the ramifications of the potential as well as the limitations for correcting heritable diseases using GM keratinocytes and/or fibroblasts.

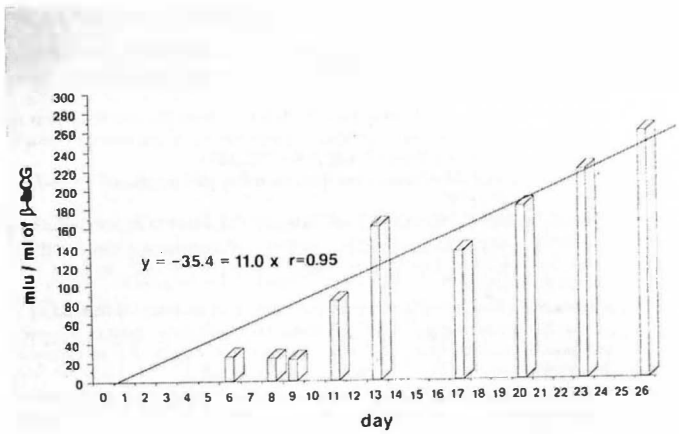

Figure 6. Production of β-hCG by human keratinocytes transduced with the β-hCG gene while being held under *in vitro* conditions for 26 d. GM keratinocytes were seeded on a collagen-glycosaminoglycans dermal substrate seeded with human fibroblasts that were not subjected to genetic modification. Media was removed about every 3 d, β-hCG measured, and new media added. There is a continual increase in the [β-hCG], this despite the fact that histologic analysis at day 26 reveals that the epidermis has matured into a stratum corneum that contains few to no nuclei.

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